Efficacy and side effects of radiation therapy in comparison with radiation therapy and temozolomide in the treatment of measurable canine malignant melanoma

Cancedda, S ; Rohrer Bley, Carla ; Aresu, L ; Dacasto, M ; Leone, V F ; Pizzoni, S ; Gracis, M ; Marconato, L

Abstract: Prognosis for unresectable canine malignant melanoma (MM) is typically poor, and therapeutic approaches remain largely palliative. A bi-institutional trial was conducted to compare efficacy and safety of radiotherapy (RT) and RT with post-radiation temozolomide in dogs with chemotherapy-naïve, measurable MM. RT consisted of 5x6 Gy fractions over 2.5 weeks. Dogs whose owners wished to pursue chemotherapy received adjuvant oral temozolomide (60 mg/m2 for 5 days every 28 days). Fifteen dogs were treated with RT only (Group 1) and 12 dogs subsequently received temozolomide (Group 2). Overall response rate was similar between Group 1 (86.7%) and Group 2 (81.1%). Median time to progression (TTP) was significantly longer in Group 2 (205 days) compared to Group 1 (110 days; p=0.046). Survival time was not significantly different between groups. Both treatments were well tolerated. Post-radiation temozolomide has a good safety profile, and may improve TTP in MM when compared to conventionally fractionated RT.

DOI: https://doi.org/10.1111/vco.12122

Efficacy and side effects of radiation therapy in comparison with radiation therapy and temozolomide in the treatment of measurable canine malignant melanoma

Simona Cancedda,[a] Carla Rohrer Bley,[b] Luca Aresu,[c] Mauro Dacasto,[c] Vito Ferdinando Leone,[a] Selene Pizzoni,[a] Margherita Gracis,[d] Laura Marconato[a,*]

a. Centro Oncologico Veterinario, Sasso Marconi, Italy
b. Division of Radiation Oncology, Vetsuisse-Faculty, University of Zurich, Switzerland
c. Department of Comparative Biomedicine and Food Science, University of Padua, Legnaro, Italy
d. Clinica Veterinaria San Siro, Milan, Italy

*** Corresponding author:**
Laura Marconato, DVM, DECVIM-CA (Oncology)
Centro Oncologico Veterinario
Via San Lorenzo 1/4
40037 Sasso Marconi, Italy
marconato@centroncologicovet.it

Key words: malignant melanoma, dog, radiation therapy, temozolomide

Abstract

Prognosis for unresectable canine malignant melanoma (MM) is typically poor, and therapeutic approaches remain largely palliative. A bi-institutional trial was conducted to compare efficacy and safety of radiotherapy (RT) and RT with post-radiation temozolomide in dogs with chemotherapy-naïve, measurable MM. RT consisted of 5x6 Gy fractions over 2.5 weeks. Dogs whose owners wished to pursue chemotherapy received adjuvant oral temozolomide (60 mg/m^2 for 5 days every 28 days). Fifteen dogs were treated with RT only (Group 1) and 12 dogs subsequently received temozolomide (Group 2). Overall response rate was similar between Group 1 (86.7%) and Group 2 (81.1%). Median time to progression (TTP) was significantly longer in Group 2 (205 days) compared to Group 1 (110 days; p=0.046). Survival time was not significantly different between groups. Both treatments were well tolerated. Post-radiation temozolomide has a good safety profile, and may improve TTP in MM when compared to conventionally fractionated RT.

Introduction

In dogs, malignant melanoma (MM) has historically been considered an extremely aggressive tumor with a high degree of local invasiveness and high metastatic propensity,[1] and is comparable to the human counterpart.[2]

While loco-regional tumor control can be achieved by means of surgery with or without radiation therapy (RT), the high metastatic potential hinders long-term control, ultimately leading to death.[3-5]

Systemic treatment of canine malignant melanoma remains suboptimal, with little evidence that chemotherapy improves survival time. Response rates to platinum compounds have traditionally been no better than 18% to 28%.[6,7] Furthermore, complete responses (CR) are rare and short in duration (3 to 5 months).[6,7] Overall, the reported long-term survival rate of canine melanoma is not encouraging due to its chemo-resistance and rapid metastasis. Furthermore, platinum compounds may cause renal and gastrointestinal toxicity, and are generally reserved for dogs with good organ function.[6,7]

Immunotherapy targeting the melanoma differentiation antigen tyrosinase has been recently explored as a strategy for the systemic treatment of canine melanoma, with contradictory results.[8,9] The combination of surgery with or without RT and the Oncept vaccine initially showed promise as superior to loco-regional treatment alone in the treatment of stage II-III canine MM,[8] until more recent studies revealed equivalent survival.[9]

Better tolerated, less toxic, and more efficacious treatments for this disease are needed. Also, because chemotherapy remains palliative, any improvement in tolerability or ease of treatment delivery is desirable.

Temozolomide, an oral alternative to the alkylating agent dacarbazine, is orally bioavailable, has greater ease of administration and lower toxicity, and has clinical activity against human melanoma equivalent to dacarbazine.[10-13] Another advantage of temozolomide over dacarbazine is its ability to undergo spontaneous conversion to the highly reactive metabolite 5-(3-methyltriazen-1-yl) imidazole-4-carboxamide without requiring any metabolic activation. Similar to dacarbazine, temozolomide also has the ability to cross the blood-brain-barrier, and may therefore play a role in treating patients with brain metastases from melanoma.[14,15] Finally, temozolomide has been shown to have radioenhancing activity in certain tumor types such as glioblastoma.[13,16]

In veterinary oncology, temozolomide has only been evaluated in dogs with lymphoma, where the combination of temozolomide and doxorubicin proved to be well tolerated and active.[17]

Aim of this prospective, controlled, non-randomized, bi-center clinical trial was to investigate objective response rate and time to progression (TTP) (primary objectives), as well as overall survival (OS) and safety profile (secondary objectives) of RT in comparison with RT and temozolomide in dogs with chemotherapy-naïve, measurable MM. It was hypothesized that the post-radiation administration of temozolomide might provide a clinical benefit over RT as sole treatment.

Material and methods

Entry requirement

Dogs with newly diagnosed or recurrent, histologically confirmed MM of any clinical stage, for which surgery was either not feasible or refused by the owners, were eligible for recruitment. Malignant melanomas included tumors arising in the oral cavity, muco-cutaneous junctions, digit, and footpad,[4,18-20] or in any other anatomic site having metastasis at presentation or high Ki-67 index (including >19.5 for oral sites, and ≥15 for cutaneous sites, as previously described).[21,22]

Work-up for all dogs included physical examination, total body computed tomography (TBCT), tumor measurement, blood cell count (CBC) with white cell differential and biochemistry, fine-needle aspirate of regional lymph nodes (including CT-guided aspiration of retro-pharyngeal nodes) regardless of their size, and fine-needle aspirate of any suspicious lesion to confirm or rule out metastasis.

All dogs were staged accordingly to the WHO clinical staging system.[23,24] For dogs undergoing chemotherapy, additional entry requirements included full recovery from RT-related acute side effects, presence of adequate organ function defined as follows: absolute neutrophil count >1500/μl, platelets >100 000/μl, creatinine level <2 mg/dl, and transaminases, bilirubin, alkaline phosphatase <1.5 upper limit of normal, and administration of at least 1 cycle of temozolomide.

Excluded from the study were dogs with any other malignancy or clinically significant comorbidity that would interfere with the study evaluation, as well as those who had received prior chemotherapy, immunotherapy and/or RT. For all dogs receiving temozolomide, owners' written consent was obtained.

Radiation therapy

Radiation was given by a Clinac DMX or Clinac iX (Varian Medical Systems, Palo Alto, CA, USA). Depending on tumor size and location, the treatment planning was performed on the basis of hand calculation for electron plans, or of a three-dimensional CT for photon plans. For treatment planning the Eclipse External Beam Planning system version 8.1 or 10.0 (Varian Oncology Systems, Palo Alto, CA, USA), respectively, was used, applying the pencil beam convolution- (version 8.6.14) or AAA-algorithm (10.0.28).

For planning CT and daily treatment, patients were placed under general anesthesia, positioned in sternal recumbency, and immobilized in an individually shaped vacuum cushion (BlueBag BodyFix, Elekta AB, Stockholm, Sweden). Dogs with tumors in the head area were additionally immobilized with a custom-made bite block.[25]

The GTV (gross tumor volume) was delineated using co-registered or in parallel viewed contrast enhanced CT images, and CTV (clinical target volume), accounting for subclinical microscopic disease extension (local microscopic disease as well as regional lymph nodes), was defined. The CTV-margin was extended three-dimensionally by 2 (for image-guided treatment) or 4 mm to define the planning target volume (PTV), accounting for internal physiologic movements, patient motion, and setup uncertainties. Organs at risk were segmented (eyes, lenses).

The prescribed dose was 30 Gy, delivered in 5 bi-weekly fractions of 6 Gy to the ICRU reference point. The recommendations for specifying dose and volumes as proposed in Reports 50 and 62 of the ICRU[26,27] were applied and variation up to +/- 7.5% within PTV was considered acceptable. Treatment was performed isocentrically with bolus and wedges to ensure dose homogeneity.

147 Radiation-related toxicity was graded according to the Veterinary Radiation Therapy
148 Oncology Group (VRTOG) scheme at each treatment, 2 weeks post completion, and
149 monthly thereafter.[28]
150
151 **Chemotherapy**
152 Adjuvant chemotherapy was offered to all dogs. If owners rejected adjuvant
153 treatment, dogs were included in Group 1. Dogs whose owners wished to pursue
154 temozolomide were included in Group 2.
155 Dogs were scheduled to start chemotherapy 2 weeks after the end of RT.
156 Temozolomide (Temozolomide capsules 20 mg, Teva, Nerviano, Italy) was
157 administered orally once daily for 5 consecutive days at a dose of 60 mg/m^2. As
158 temozolomide in Italy only comes as 5-mg and 20-mg capsules, the dose was
159 administered to the nearest 5 mg. Treatment cycles were repeated every 28 days for
160 4 cycles.
161 If there was evidence of tumor shrinkage or stable disease (SD) at the end of the first
162 2 cycles, dogs continued on to the next 2 cycles. If at any time there was evidence of
163 disease progression or toxicity, temozolomide was discontinued and other options
164 were discussed with the owners.
165 All dogs were given prophylactic 25 mg kg^{-1} BID oral clavulanate-potentiated
166 amoxicillin (Synulox tablets 500 mg, Pfizer, Rome, Italy) for 7 days after each of the
167 first 2 treatments, and only if needed thereafter. Standard antiemetic therapy with oral
168 2 mg kg^{-1} maropitant (Cerenia tablets 60 mg, Pfizer, Rome, Italy) was administered if
169 necessary.
170 Toxicity resulting from temozolomide was assessed based on the dog history,
171 physical examination and CBC before the beginning of each next cycle and 10 days

after the administration, as reported by the Veterinary Co-operative Oncology Group.[29] In case of grade 2 toxicities, therapy was delayed for 1 week until < grade 2, then restarted at the same dose. Conversely, the dose was reduced by 25% of the starting dose when grade 3 or 4 hematological or non-hematological toxicity occurred.

Treatment response

In either Group, tumor response was evaluated monthly by physical examination, chest x-ray, TBCT, or other diagnostic tests (including fine-needle aspiration of the regional lymph nodes), as appropriate. Standard Response Evaluation Criteria In Solid Tumors (RECIST) were used for response.[30]

In particular, for dogs with oral melanoma (regardless of clinical stage) or melanoma at any site with distant metastasis, a TBCT was repeated at the end of RT. For melanoma arising at other anatomical sites without distant metastasis, dimensions of the primary tumor were manually measured, and distant metastasis were ruled out by thoracic radiographs and abdominal ultrasound.

Complete remission was defined as the disappearance of all target lesions. Partial response (PR) was defined as a reduction of at least 30% in the sum of diameters of target lesions from baseline. Stable disease (SD) was defined as < 30% decrease or >20% increase in sum of diameters of target lesions from smallest sum while on treatment. Progressive disease (PD) was defined as an increase in the sum of diameters of target lesions by at least 20% over the size present at entry on study, or the appearance of new lesions. Responses were required to last for at least 28 days. Once PD was documented, rescue therapy of any kind was allowed.

Statistical analysis

Dogs in Group 1 were assessed for response and toxicity if they completed the RT protocol, while those in Group 2 if they had received at least one cycle of treatment.

In both groups, the overall response rate was defined as the number of dogs achieving CR or PR, compared with the total number of dogs treated. When considering the efficacy of RT, local disease was measured to assess degree of response. When considering the effect of temozolomide, both local and distant disease was measured.

TTP was calculated from the date of initiation of treatment to the first progression of the disease (local and/or distant, depending on the treatment arm). TTP was censored for dogs without evidence of PD when lost to follow-up or at death. Dogs that died due to disease-related factors without having previously documentation of PD were considered as an event at the estimation of TTP.

Survival time was calculated from the date of initiation of treatment to the date of death or day of last follow-up. Survival was censored for dogs alive at study's end, dogs lost to follow-up, and dogs with a cause of death unrelated to MM or therapy for MM. If the cause of death was not known, death was attributed to MM.

Fisher exact test was used to compare dogs' characteristics at baseline, treatment response and toxicity. The Kaplan–Meier method was used to calculate TTP and OS curves, while the log-rank test was used to compare time to event distributions. The analysis was made on an intent-to-treat basis. A p-value of < 0.05 was considered significant for all analysis. All analyses were performed with a standard statistical software (GraphPad Prism, GraphPad La Jolla, CA, USA).

Results

Dogs and tumor characteristics

Between July 2009 and November 2013, 27 dogs met the inclusion criteria and were enrolled at 2 centers (Centro Oncologico Veterinario, Sasso Marconi, Italy, and Division of Radiation Oncology, Vetsuisse-Faculty, Zurich, Switzerland). Of those, 15 dogs were treated with RT (Group 1) and 12 dogs were irradiated and subsequently treated with temozolomide (Group 2).

In Group 1, there were 4 mixed-breed dogs and one each of the following: Great Dane, English Cocker Spaniel, Pug, Rottweiler, Dachshund, Pekingese, Italian Bracco, Poodle, Border collie, Chow Chow and Viszla. There were 8 males (of which 3 castrated), and 7 spayed females. Median age was 13 years (range, 8 to 14 years), and median weight was 22 kg (range, 5.5 to 60.2 kg).

MM location included mandible (n=6), maxilla (n=5), and one each of the following: eyelid, nasal planum, axilla, and footpad. Overall, 1 had stage I disease, 1 had stage II disease, 10 had stage III disease, and 3 had stage IV disease. Metastatic location for dogs with oral melanoma was as follows: bilateral mandibular lymph nodes (n=2), ipsilateral mandibular lymph node (n=1), lungs (n=1), ipsilateral mandibular and retropharyngeal lymph nodes and lungs (n=1). The dog with a palpebral MM had metastatic ipsilateral mandibular lymph node, the dog with nasal MM had metastatic bilateral mandibular lymph nodes, and the dog with MM on his paw had metastatic ipsilateral cervical lymph node. The dog with stage I oral melanoma and the one with

stage III cutaneous melanoma had a Ki67 index of 23% and 18%, respectively, and were both considered to be biologically aggressive.

Two dogs had previous surgeries and presented with macroscopic recurrent disease. Thirteen dogs had inoperable MM at first presentation.

In Group 2, there were 4 mixed-breed dogs, and one each of the following: Dogue de Bordeaux, Schnauzer, Hound, Labrador retriever, Spitz, Golden retriever, German shepherd and Dachshund. There were 9 males (of which 2 castrated) and 3 females (of which one spayed). Median age was 13 years (range, 7 to 15 years), and median weight was 29 kg (range, 3.5 to 44.5 kg).

MM location included maxilla (n=7), mandible (n=2), eyelid (n=1), digit (n=1), and mucosal aspect of the lip (n=1). Overall, 1 dog had stage I disease, 2 had stage II disease, 8 had stage III disease, and 1 had stage IV disease. Metastatic location for dogs with oral melanoma was as follows: ipsilateral mandibular lymph node (n=2), bilateral mandibular lymph nodes (n=1), and ipsilateral mandibular lymph node and lungs (n=1). The dog with a palpebral MM had metastatic bilateral mandibular lymph nodes, and the dog with a digit melanoma had a metastatic ipsilateral prescapular lymph node. The dog with stage I oral melanoma had a Ki67 index of 28%, and was therefore included.

Seven dogs had previous surgeries, whereas for 5 dogs presented with naïve disease.

Dogs' demographics were similar for each treatment group (Table 1). There were no significant differences in breed, age, sex, disease site, and distribution of stage at baseline between the treatment groups. Rates of previous surgeries were statistically

different across groups, due to the higher percentage of dogs undergoing previous excision in Group 2 compared to Group 1 ($p = 0.013$).

Treatment and toxicity

All dogs in Group 1 completed the planned RT protocol within 2.5 weeks. Toxicity occurred in 7 dogs and was limited to grade 1-2 mucositis, which resolved uneventfully within 3 weeks after the end of RT.

Similarly, all dogs in Group 2 completed the planned RT protocol within 2.5 weeks. Toxicity occurred in 4 dogs and was limited to grade 1-2 mucositis. Two to 3 weeks after RT, this group of dogs received 1 to 4 (median, 4) cycles of temozolomide, with 6 (50%) dogs receiving 4 cycles, 3 (25%) receiving 3 cycles, 2 (16.7%) receiving 2 cycles, and 1 (8.3%) dog receiving 1 cycle only. Reasons for not completing the planned 4 cycles were PD (n=4), financial concern (n=1) and melanoma-unrelated death (n=1). Chemotherapy was well tolerated, resulting in no dose reduction. Grade 1 gastrointestinal toxicity consisting of nausea and loss of appetite occurred in 4 (33.3%) dogs after the first cycle. No dogs stopped treatment because of toxicity, and no dog died due to complications of chemotherapy.

Response rate and time to progression

The primary melanomas from 13 dogs in Group 1 responded to RT, with an overall local response rate of 86.7% and a median TTP of 110 days (range, 60 to 798 days; 95% CI 65-155). Among responders, 3 dogs obtained CR for 124, 174 and 798 days, and 10 dogs achieved PR with a median duration of 95 days (range, 29 to 236 days). Two dogs had SD for 34 and 59 days, respectively. At data analysis closure, all dogs

had documented disease progression: 8 dogs had local failure, 3 had distant failure, and 4 dogs had local and distant failure. Four dogs in this group developed distant metastasis during the study period: 2 of them developed lung metastasis, 1 dog developed pulmonary and brain metastasis, and 1 dog developed brain metastasis after a median of 87 days from the initial presentation (range, 39 to 165 days).

Regarding Group 2, at the end of RT and before starting chemotherapy, 2 dogs obtained CR, and 8 dogs obtained PR, with an overall local response rate to RT of 83.3%. Two dogs obtained SD. When considering the effect of temozolomide, the overall (local and distant) response rate was of 81.1% and the median TTP was 205 days (range, 55 to 1010 days; 95% CI 141.9-242.1). Two dogs obtained CR for 1010 and 205 days, 8 dogs obtained PR for a median of 138 days (range, 55 to 181 days), and 2 dogs had SD for 78 and 143 days.

At data analysis closure, 6 dogs had documented disease progression: 1 had local failure, and 5 had distant failure. During the study period, 4 dogs developed distant metastasis to lung (n=3) and bone (n=1) after 55, 78, 88 and 181 days.

Treatment response rate and distant metastatic rate were not significantly different between groups (Table 2). However, dogs in Group 2 had a significantly longer TTP than dogs in Group 1 ($p = 0.046$; Fig. 1). Also, 6 (50%) of the dogs in Group 2 experienced disease progression, compared with 15 (100%) dogs in Group 1 ($p = 0.001$).

Rescue treatment

Overall, 3 dogs received rescue treatment after PD was documented. In Group 1, one dog underwent surgical excision after local failure and died after 196 days from initial presentation, and 1 dog received a different investigational cytotoxic therapy

and died after 165 days. In Group 2, 1 dog was irradiated again after local failure, and was still alive 596 days from initial presentation.

Survival

At the end of the study, 14 dogs in Group 1 were dead and 1 (with stage I disease) was still alive after 187 days. Among the dead dogs, 2 died for tumor-unrelated disease; however in both dogs the melanoma was locally progressing. In the remaining dogs, death was attributable to local failure (n=5), local and distant failure (n=4), and distant failure (n=3). In Group 2, 8 dogs had died and 4 (3 with stage III disease, and 1 with stage II disease) were still alive with a median follow-up of 405 days. Three dogs died for tumor-unrelated causes (2 with PR and 1 with SD). In the remaining 5 dogs, death was attributable to distant failure. Survival did not differ significantly between the 2 groups (192 days for Group 1 versus 401 days for Group 2, $p = 0.093$; Table 2).

Discussion

Advanced melanoma represents one of the most treatment-refractory malignancies and its treatment remains unsatisfactory. Despite decades of research, there have been no new drugs advisable for use in canine melanoma, and a consensus on a standard first-line treatment has yet to be established.

The median survival for dogs with surgically resected stage II-III oral melanoma is only 3–12 months with an estimated 1-year survival rate of approximately 20%.[31,32]

Regardless of the clinical stage, a threshold for Ki67 above 19.5 predicted melanoma-related death by 1-year post diagnosis, and should therefore be considered as a negative prognostic factor.[21] For cutaneous MM, a high Ki67 value has also been associated with shorter survival.[22]

Unfortunately, these survival figures have not changed in more than a decade of clinical studies, mostly due to the unsatisfactory efficacy of systemic chemotherapy,[6,33,34] and even to newer treatment options, such as gene therapy.[35,36] Equally important, the role of new treatment modalities (e.g. immunotherapy) in influencing outcome of MM trials has been conflicting, and promising results of single-institution studies have repeatedly failed to survive the scrutiny of subsequent studies.[9,37] Therefore, loco-regional therapy with palliative intent remains the current mainstay of treatment.

The results of this study showed that dogs with measurable MM that received post-radiation temozolomide might have a prolonged TTP compared with dogs undergoing RT only.

On an intent-to-treat basis the overall response rates were similar for both treatments arms (81-86%), being in agreement with the previous literature documenting CR rates of 53-69% and PR rates of 25-30% in dogs receiving coarse fractionation RT schemes.[34,38-41] However, dogs treated with post-RT temozolomide had a median TTP of 205 days compared to 110 days in dogs undergoing RT only, and this difference was statistically significant. Also, dogs receiving temozolomide had a significant lower local and distant failure rate compared with irradiated dogs (50% versus 100%, respectively).

The median survival time in Group 1 and Group 2 of 192 and 401 days, respectively, is comparable to that reported in the literature describing RT with or without carboplatin.[34,42] Although dogs receiving temozolomide lived longer that irradiated dogs (401 and 192 days, respectively), this difference only tended to be statistically significant. This result may be partly attributable to the small sample size and to the insufficient follow-up interval such that a survival advantage could not be discerned. Indeed, 4 dogs in Group 2 were still alive at data analysis closure, compared to 1 dog in Group 1. It may be possible that a longer follow-up might translate into a survival benefit as well.

Many variables have been previously shown to have prognostic value in dogs with MM, including anatomical site, tumor size, bone lysis, and clinical stage.[4,19,34] To be representative of the general melanoma population, dogs with high-risk non-oral MM were included in this study. It was felt that these dogs had the potential to benefit from systemic chemotherapy because of the documented metastasis at initial presentation. The dogs in our study had comparable baseline characteristics between the treatment arms, demonstrating no imbalance in known prognostic factors. Dogs receiving temozolomide were more likely to have undergone previous surgery compared with irradiated dogs, and this difference was significant. This could be a random finding, or alternatively be due to a selection bias resulting in owners being more likely to pursue further treatment after already having agreed on multiple surgeries.

In the present study, long-term survival did not occur, with the exception of 3 dogs achieving an initial CR in response to RT; 2 of them additionally received post-

radiation temozolomide. All of them had high-risk features at diagnosis, including bilateral regional lymph nodes metastasis (n=2), and a T3 oral tumor (n=1). One of them died because of melanoma-unrelated causes after 826 days, and the 2 other dogs are still alive after 1041 and 635 days, one of them still being in durable first CR. Although we did not look statistically at this, it may be possible that achievement of CR after RT may translate into prolonged survival.

Temozolomide, which is orally formulated, allows for outpatient treatment. This is particularly desirable for dogs with a tended short life expectancy. In this case series, treatment was generally well tolerated; the reported adverse events were consistent with prior RT reports, and no new clinically significant temozolomide safety issues were identified in the present study. Nausea and loss of appetite were reported in the temozolomide group, which is consistent with the prescribing information for this agent.[43] Hematologic toxicities were not an issue with temozolomide.

The toxicity profile of temozolomide in this trial therefore appears to be manageable. However, it is possible that the acceptable tolerability of temozolomide in this study may be a consequence of underdosing and could therefore explain the observed nonsignificant metastatic rate differences between groups. Indeed, the number of dogs that developed distant metastasis in Group 1 (4 of 15, 26.7%) was comparable to Group 2 (4 of 12, 33.3%),. Notably, no dogs in Group 2 developed symptomatic cerebral metastasis, in contrast to Group 1 (2 of 4, 50%), suggesting that temozolomide may prevent the occurrence of metastasis to the brain. In dogs, temozolomide has only been used in combination with doxorubicin to treat lymphoma,[17] and no Phase I dose-escalation clinical trials have been conducted so far. In this previously published paper, the starting dose of oral temozolomide was 60

420 mg/m^2 administered every 24 hours for 5 consecutive days. This dose was elected
421 on the basis of available toxicology data for dogs,[44] the minimum risk of side effects
422 when administered at the same dose in humans, and the convenience of
423 administration given the currently available capsule sizes. Temozolomide was
424 escalated up to a median dose of approximately 90 mg/m^2 with various grades of
425 hematological, gastrointestinal and renal toxicity. Of note, the authors reported no
426 significant association between temozolomide dose and duration of response.[17]
427 Based on the above, in the current study the dose of 60 mg/m^2 was elected.
428 Temozolomide is considered to be a radiosensitizing agent for certain tumor
429 types,[13,16] and at present there are no studies in veterinary medicine exploring the
430 possible overlapping toxicity profile of this drug given after RT. Furthermore, since
431 chemotherapy was intended to be palliative, it was decided to use a dose that was
432 considered to be safe. Indeed, treatment was well tolerated, with gastrointestinal
433 toxicity no higher than grade 1 reported as the only adverse event. It is therefore
434 likely that the dose of temozolomide used here was overcautious with regard to
435 toxicity and that the maximal dosage range was not explored in full. In people, it has
436 been shown that extended dosing regimens allow for administration of a higher
437 cumulative dose per cycle, thereby depleting O6-methylguanine-DNA
438 methyltransferase and consequently enhancing cytotoxic activity.[45]
439
440 The prophylactic use of antibiotics in patients with cancer undergoing chemotherapy is
441 controversial.[46,47] However, it is well documented that infections are significant
442 causes of morbidity and mortality among immunocompromised patients. Oral and
443 digital tumors are often ulcerated, providing a source of microbial infections.
444 Furthermore, RT-related side effects also remain a major source of illness, as

radiation injury decreases host defenses.[48] Although temozolomide was not associated with significant hematological toxicity, prophylactic antibiotics were administered during the first cycles of chemotherapy aiming at reducing the risk of infection associated with cancer itself, RT or chemotherapy-related neutropenia.

This study has some limitations. Although it was attempted to have a homogeneous population of high-risk tumors, dogs with melanoma arising at different anatomic sites and of different clinical stages were enrolled, possibly having increased the biologic variability in this study. Also, because of the small sample size included in the present study, it is not possible to know whether TTP and survival time among a larger number of dogs treated in a similar fashion would be similar. Another limitation of this study is the lack of randomization. It may be possible that pet owners whose dogs had a less advanced clinical stage were more motivated to participate in a clinical trial. Finally, methylation of MGMT has been associated with greater benefit from temozolomide that unmethylated MGMT in people with unresectable glioblastoma.[49] Additional molecular, biological or host factors need to be identified in dogs to predict tumor response to chemotherapy.

In conclusion, coarse fractionated RT followed by temozolomide may improve TTP compared with RT alone in dogs with chemotherapy-naïve, measurable MM. The good safety profile, and ease of administration suggest that temozolomide could play an important role in the future management of this disease. Further clinical research is warranted to refine the choice of temozolomide dose.

References

1. Smedley RC, Spangler WL, Esplin DG, Kitchell BE, Bergman PJ, Ho HY, Bergin IL and Kiupel M. Prognostic markers for canine melanocytic neoplasms: a comparative review of the literature and goals for future investigation. *Veterinary Pathology* 2011; **48**: 54-72.
2. Gillard M, Cadieu E, De Brito C, Abadie J, Vergier B, Deavauchelle P, Degorce F, Dréano S, Primot A, Dorso L, Lagadic M, Galibert F, Hédan B, Galibert MD, André C. Naturally occurring melanomas in dogs as models for non-UV pathways of human melanomas. *Pigment Melanoma Research* 2014; **27**: 90-102.
3. Bolon B, Calderwood Mays MB and Hall BJ. Characteristics of canine melanomas and comparison of histology and DNA ploidy to their biologic behavior. *Veterinary Pathology* 1990; **27**: 96–102.
4. Spangler WL and Kass PH. The histologic and epidemiologic bases for prognostic considerations in canine melanocytic neoplasia. *Veterinary Pathology* 2006; **43**:136-149.
5. Schultheiss PC. Histologic features and clinical outcomes of melanomas of lip, haired skin, and nail bed locations of dogs. *Journal of Veterinary Diagnostic Investigations* 2006; **18**: 422–425.
6. Rassnick KM, Ruslander DM, Cotter SM, Al-Sarraf R, Bruyette DS, Gamblin RM, Meleo KA and Moore AS. Use of carboplatin for treatment of dogs with malignant melanoma: 27 cases (1989-2000). *Journal of the American Veterinary Medical Association* 2001; **218**: 1444-1448.
7. Boria PA, Murry DJ, Bennett PF, Glickman NW, Snyder PW, Merkel BL, Schlittler DL, Mutsaers AJ, Thomas RM and Knapp DW. Evaluation of cisplatin combined with piroxicam for the treatment of oral malignant melanoma and

494 oral squamous cell carcinoma in dogs. *Journal of the American Veterinary*
495 *Medical Association* 2004; **224**: 388-394.
496 8. Bergman PJ, McKnight J, Novosad A, Charney S, Farrelly J, Craft D, Wulderk
497 M, Jeffers Y, Sadelain M, Hohenhaus AE, Segal N, Gregor P, Engelhorn M,
498 Riviere I, Houghton AN and Wolchok JD. Long-term survival of dogs with
499 advanced malignant melanoma after DNA vaccination with xenogeneic human
500 tyrosinase: a phase I trial. *Clinical Cancer Research* 2003; **9**: 1284-1290.
501 9. Ottnod JM, Smedley RC, Walshaw R, Hauptman JG, Kiupel M and
502 Obradovich JE. A retrospective analysis of the efficacy of Oncept vaccine for
503 the adjunct treatment of canine oral malignant melanoma. *Veterinary and*
504 *Comparative Oncology* 2013; **11**: 219-229.
505 10. Middleton MR, Grob JJ, Aaronson N, Fierlbeck G, Tilgen W, Seiter S, Gore M,
506 Aamdal S, Cebon J, Coates A, Dreno B, Henz M, Schadendorf D, Kapp A,
507 Weiss J, Fraass U, Statkevich P, Muller M and Thatcher N. Randomized
508 phase III study of temozolomide versus dacarbazine in the treatment of
509 patients with advanced metastatic melanoma. *Journal of Clinical Oncology*
510 2000; **18**: 158-166.
511 11. Bleehen NM, Newlands ES, Lee SM, Thatcher N, Selby P, Calvert AH, Rustin
512 GJ, Brampton M and Stevens MF. Cancer Research Campaign phase II trial
513 of temozolomide in metastatic melanoma. *Journal of Clinical Oncology* 1995;
514 **13**: 910-913.
515 12. Quirt I, Verma S, Petrella T, Bak K and Charette M. Temozolomide for the
516 treatment of metastatic melanoma: a systematic review. *The Oncologist* 2007;
517 **12**: 1114-1123.

13. Kil WJ, Cerna D, Burgan WE, Beam K, Carter D, Steeg PS, Tofilon PJ and Camphausen K. In vitro and in vivo radiosensitization induced by the DNA methylating agent temozolomide. *Clinical Cancer Research* 2008; **14**: 931-938.
14. Song RB, Vite CH, Bradley CW and Cross JR. Postmortem evaluation of 435 cases of intracranial neoplasia in dogs and relationship of neoplasm with breed, age, and body weight. *Journal of Veterinary Internal Medicine* 2013; **27**: 1143-1152.
15. Snyder JM, Lipitz L, Skorupski KA, Shofer FS and Van Winkle TJ. Secondary intracranial neoplasia in the dog: 177 cases (1986-2003). *Journal of Veterinary Internal Medicine* 2008; **22**: 172-177.
16. Chakravarti A, Erkkinen MG, Nestler U, Stupp R, Mehta M, Aldape K, Gilbert MR,Black PM and Loeffler JS. Temozolomide-mediated radiation enhancement in glioblastoma: a report on underlying mechanisms. *Clinical Cancer Research* 2006; **12**: 4738-4746.
17. Dervisis NG, Dominguez PA, Sarbu L, Newman RG, Cadile CD, Swanson CN and Kitchell BE. Efficacy of temozolomide or dacarbazine in combination with an anthracycline for rescue chemotherapy in dogs with lymphoma. *Journal of the American Veterinary Medical Association* 2007; **231**: 563-569.
18. Goldschmidt MH. Benign and malignant melanocytic neoplasms of domestic animals. *American Journal of Dermatopathology* 1985; **7**: 203-212.
19. Harvey HJ, MacEwen EG, Braun D, Patnaik AK, Withrow SJ and Jongeward S. Prognostic criteria for dogs with oral melanoma. *Journal of the American Veterinary Medical Association* 1981; **178**: 580-582.

20. Bostock DE. Prognosis after surgical excision of canine melanomas. Veterinary Pathology 1979; 16: 32-40.
21. Bergin IL, Smedley RC, Esplin DG, Spangler WL, Kiupel M. Prognostic evaluation of Ki67 threshold value in canine oral melanoma. *Veterinary Pathology* 2011; **48**: 41-53.
22. Laprie C, Abadie J, Amardeilh MF, Net JL, Lagadic M and Delverdier M. MIB-1 immunoreactivity correlates with biologic behaviour in canine cutaneous melanoma. *Veterinary Dermatology* 2001; **12**: 139-147.
23. Owens L. TNM classification of tumors in domestic animals. *World Health Organization Bulletin.* Geneva: 1980; 23.
24. Manley CA, Leibman NF, Wolchok JD, Rivière IC, Bartido S, Craft DM and Bergman PJ. Xenogeneic murine tyrosinase DNA vaccine for malignant melanoma of the digit of dogs. *Journal of Veterinary Internal Medicine* 2011; **25**: 94-99.
25. Rohrer Bley C, Blattmann H, Roos M, Sumova A and Kaser-Hotz B. Assessment of a radiotherapy patient immobilization device using single plane port radiographs and a remote computed tomography scanner. *Veterinary Radiology & Ultrasound* 2003; **44**: 470–475.
26. International Commission of Radiation Units and Measurements. Prescribing, recording, and reporting photon beam therapy, ICRU Report 50. Bethesda, MD: ICRU, 1993.
27. International Commission of Radiation Units and Measurements. Prescribing, recording, and reporting photon beam therapy (Supplement to ICRU Report 50), ICRU Report 62. Bethesda, MD: ICRU, 1999.

28. Ladue T, Klein MK and Veterinary Radiation Therapy Oncology Group. Toxicity criteria of the veterinary radiation therapy oncology group. *Veterinary* Radiology & Ultrasound 2001; **42**: 475–476.
29. Veterinary Cooperative Oncology Group. Veterinary cooperative oncology group- common terminology criteria for adverse events (VCOG-CTCAE) following chemotherapy or biological antineoplastic therapy in dogs and cats v1.0. *Veterinary and Comparative Oncology* 2004; **2**: 194-213.
30. Nguyen SM, Thamm DH, Vail DM and London CA. Response evaluation criteria for solid tumours in dogs (v1.0): a Veterinary Cooperative Oncology Group (VCOG) consensus document. *Veterinary and Comparative Oncology* 2013 Mar 28. doi: 10.1111/vco.12032.
31. MacEwen EG, Patnaik AK, Harvey HJ, Hayes AA and Matus R. Canine oral melanoma: comparison of surgery versus surgery plus Corynebacterium parvum. *Cancer Investigation* 1986; **4**: 397-402.
32. Grosenbaugh DA, Leard AT, Bergman PJ, Klein MK, Maleo K, Susaneck S, Hess PR, Jankowski MK, Jones PD, Leibman NF, Johnson MH, Kurzman ID and Wolchok JD. Safety and efficacy of a xenogeneic DNA vaccine encoding for human tyrosinase as adjunctive treatment for oral malignant melanoma in dogs following surgical excision of the primary tumor. *American Journal of Veterinary Research* 2011; **72**: 1631–1638.
33. Brockley LK, Cooper MA and Bennett PF. Malignant melanoma in 63 dogs (2001-2011): the effect of carboplatin chemotherapy on survival. *New Zealand Veterinary Journal* 2013; **61**: 25-31.
34. Proulx DR, Ruslander DM, Dodge RK, Hauck ML, Williams LE, Horn B, Price GS and Thrall DE. A retrospective analysis of 140 dogs with oral melanoma

591 treated with external beam radiation. *Veterinary Radiology & Ultrasound* 2003;
592 **44**: 352-359.
593 35. Westberg S, Sadeghi A, Svensson E, Segall T, Dimopoulou M, Korsgren O,
594 Hemminki A, Loskog AS, Tötterman TH and von Euler H. Treatment efficacy
595 and immune stimulation by AdCD40L gene therapy of spontaneous canine
596 malignant melanoma. *Journal of Immunotherapy* 2013; **36**: 350-358.
597 36. Murakami A, Mori T, Sakai H, Murakami M, Yanai T, Hoshino Y, Maruo K.
598 Analysis of KIT expression and KIT exon 11 mutations in canine oral
599 malignant melanomas. *Veterinary and Comparative Oncology* 2011; **9**: 219-
600 224.
601 37. Herzog A, Buchholz J, Ruess-Melzer K, Lang J, Kaser-Hotz B. Concurrent
602 irradiation and DNA tumor vaccination in canine oral malignant melanoma: a
603 pilot study. *Schweizer Archiv fur Tierheilkunde* 2013; **155**: 135-142.
604 38. Bateman KE, Catton PA, Pennock PW, Kruth SA. 0-7-21 radiation therapy for
605 the treatment of canine oral melanoma. *Journal of Veterinary Internal Medicine*
606 1994; **8**: 267-272.
607 39. Blackwood L, Dobson JM. Radiotherapy of oral malignant melanomas in dogs.
608 *Journal of the American Veterinary Medical Association* 1996; **209**: 98-102.
609 40. Théon AP, Rodriguez C, Madewell BR. Analysis of prognostic factors and
610 patterns of failure in dogs with malignant oral tumors treated with megavoltage
611 irradiation. *Journal of the American Veterinary Medical Association* 1997; **210**:
612 *778-784.*
613 41. Freeman KP, Hahn KA, Harris FD, King GK. Treatment of dogs with oral
614 melanoma by hypofractionated radiation therapy and platinum-based
615 chemotherapy (1987-1997). *Journal of Veterinary Internal Medicine* 2003; **17**:

96-101.

42. Murphy S, Hayes AM, Blackwood L, Maglennon G, Pattinson H, Sparkes AH. Oral malignant melanoma - the effect of coarse fractionation radiotherapy alone or with adjuvant carboplatin therapy. *Veterinary and Comparative Oncology* 2005; **3**: 222-229.
43. Newlands ES, Blackledge GR, Slack JA, Rustin GJ, Smith DB, Stuart NS, Quarterman CP, Hoffman R, Stevens MF, Brampton MH, et al. Phase I trial of temozolomide (CCRG 81045: M&B 39831: NSC 362856). *British Journal of Cancer* 1992; **65**: 287-291.
44. Initial scientific discussion for the approval of Temodal. European Medicines Agency Web site. Available at: www.emea.europa.eu/humandocs/PDFs/EPAR/Temodal/274198en6.pdf. Accessed Jan 1, 2007.
45. Neyns B, Tosoni A, Hwu WJ, Reardon DA. Dose-dense temozolomide regimens: antitumor activity, toxicity, and immunomodulatory effects. Cancer 2010; **116**: 2868-2877.
46. Saloustros E, Tryfonidis K, Georgoulias V. Prophylactic and therapeutic strategies in chemotherapy-induced neutropenia. *Expert Opinion on Pharmacotherapy* 2011; **12**: 851-863.
47. Chretin JD, Rassnick KM, Shaw NA, Hahn KA, Ogilvie GK, Kristal O, Northrup NC, Moore AS. Prophylactic trimethoprim-sulfadiazine during chemotherapy in dogs with lymphoma and osteosarcoma: a double-blind, placebo-controlled study. *Journal of Veterinary Internal Medicine* 2007; **21**: 141-148.
48.
49. Stone HB, Coleman CN, Anscher MS, McBride WH. Effects of radiation on normal tissue: consequences and mechanisms. *Lancet Oncology* 2003; **4**:

529-536.Capdevila L, Cros S, Ramirez JL, Sanz C, Carrato C, Romeo M, Etxaniz O, Hostalot C, Massuet A, Cuadra JL, Villà S, Balañà C. Neoadjuvant cisplatin plus temozolomide versus standard treatment in patients with unresectable glioblastoma or anaplastic astrocytoma: a differential effect of MGMT methylation. *Journal of Neuro-oncology* 2014; **117**: 77-84.

Figure legend

Fig 1. Kaplan–Meier curve depicting TTP of dogs treated with RT (blue line) and with RT and temozolomide (green line).